*PENICILLINASE PLASMID DNA FROM STAPHYLOCOCCUS AUREUS**

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Abstract.—A penicillinase plasmid from *Staphylococcus aureus* and three of its derivatives, all previously identified as extrachromosomal genetic elements, have been isolated in high yield as circular duplex DNA molecules. The wild-type plasmid was found by contour-length measurements of electron micrographs to have a molecular weight of 18.6×10^6 daltons. Two plasmids with deletions encompassing six and eight of the eleven known plasmid cistrons had molecular weights of 16.4×10^6 and 15.3×10^6 daltons, respectively. This information was used to establish approximate physical distances for the genetic map. A high-frequency transducing element also derived from the plasmid had a molecular weight of approximately 24×10^6 daltons. Although each plasmid preparation appeared homogeneous by ultracentrifugal analysis, electron micrographs always revealed the presence of a low percentage of complex oligomeric forms, particularly circular and catenated dimers.

There is considerable evidence indicating that many strains of *Staphylococcus aureus* harbor extrachromosomal factors responsible for resistance to penicillin.[1, 2] These elements, collectively known as penicillinase plasmids, have been found to contain structural and control genes for penicillinase[1] as well as genes for resistance to erythromycin[3] and to a series of inorganic ions.[4, 5] On the plasmid genetic map, the resistance genes appear to be grouped, and topographically separate from a region essential for plasmid autonomy—a region involved in plasmid maintenance, compatibility, and replication (*mcr*).[6]

Deletions of plasmid segments occasionally occur as a consequence of plasmid transfer by transduction.[6, 7] Examination of residual markers for a series of independent deletions of the same parental plasmid, PI_{258}, has permitted the construction of a linear deletion map with the *mcr* region at one end and the marker for the locus of erythromycin resistance (*ero*) at the other.[6] Since linkage between the two end markers has been demonstrated in recombination studies,[6] the over-all genetic map, as shown in Figure 1, is circular.[8]

In addition to deletions, a high-frequency transducing element, P11*de*, has also been studied. In this element a large segment of the plasmid genome has been replaced by a section of the genome of the transducing phage, P11.[9] The phage moiety of this derivative element is cryptic, but is demonstrable by its ability to complement and rescue markers from superinfecting P11 mutants. Although strains harboring P11*de* give rise to high-frequency transducing lysates for erythromycin resistance after superinfection with P11, the composite element appears to lead an autonomous, plasmid-like existence.[9]

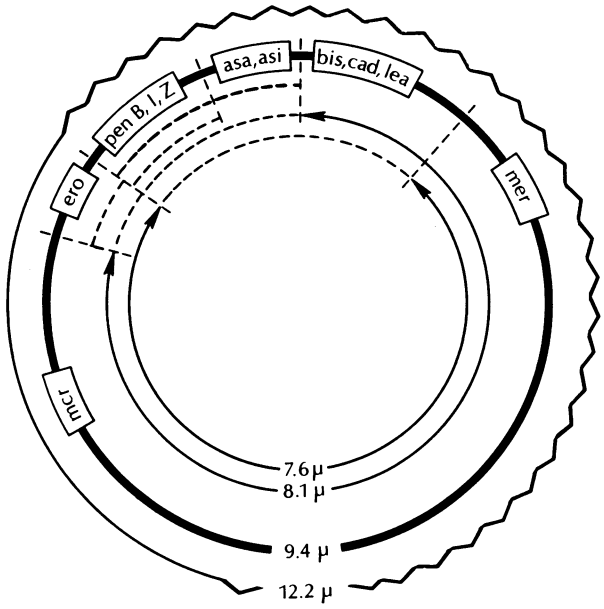

FIG. 1.—The circular genetic map of a penicillinase plasmid. Although the sequence of genetic markers is assumed to be accurate, the relative distances between them are approximate and are based primarily on the contour lengths of plasmid deletions presented in Table 1. The extent and location of these deletions and others used to establish marker orders are represented on the genetic map as dashed lines. The genome of the wild-type plasmid, PI_{258}, including all of its known markers is shown as a heavy circle. *Pen*B, *pen*I, *pen*Z are two control loci[23] and the structural locus for penicillinase, respectively; *asa*, *asi*, *bis*, *cad*, *lea*, and *mer* are determinants of resistance to arsenate, arsenite, bismuth, cadmium, lead, and mercuric ions, respectively; *ero* is a locus for erythromycin resistance and *mcr* is a region involved in plasmid maintenance, compatibility, and replication.[2, 5, 6] Thus, deletion PI_{258} *pen* 102 (contour length 7.6 μ) lacks markers *pen*B, *pen*I, *pen*Z, *asa*, *asi*, *bis*, *cad*, and *lea*, while deletion $P11_{147}$ *ero* 15 (contour length 8.1 μ) lacks markers *ero*, *pen*B, *pen*I, *pen*Z, *asa*, and *asi*. P11*de* (12.2 μ) lacks all markers except *mcr* and *ero*. The wavy line represents material derived from the phage genome.

We report here the isolation of covalently closed, circular DNA from four strains of *Staphylococcus* harboring, respectively, the wild-type parental plasmid (PI_{258}), two of its deletions, and P11*de*. The molecular weights of these four species of circular DNA are correlated with the genetic lengths of the respective elements and permit the construction of a partial physical map.

Materials and Methods.—Bacterial strains: The host strain in all cases was RN450, an apparently nonlysogenic derivative of 8325,[9] which does not harbor any known penicillinase plasmid. RN453 harbors the wild-type plasmid, PI_{258}, transduced from strain MS258.[6, 7] RN455 contains the defective high-frequency transducing element, P11*de*, derived from PI_{258}.[9] RN494 and RN893 harbor derivative plasmids with extensive deletions, PII_{147} *ero* 15 and PI_{258} *pen* 102, respectively. The former is derived from a recombinant plasmid whose parents were PI_{258} and PI_{147};[6, 7] the latter is derived from PI_{258}. The plasmid nomenclature is described by Peyru *et al.*[2]

Preparations: Ethidium bromide was obtained from Boots Pure Drug Co., Ltd.; RNase from Sigma Chemical Co.; lysostaphin was a gift of Mead Johnson and Company.

RNase stock solutions, containing 1 mg/ml of nuclease in distilled water, were heated to 80° for 5 min prior to use.

Nitrocellulose chromatography: Hercules nitrocellulose "cubed 1/4 SEC" was obtained from Randolph Products Co., and was thoroughly ground in 2 × SSC[10] with a mortar and pestle. The ground nitrocellulose was packed under pressure (3 psi) in a 2-cm diameter column to a final bed volume of 30 ml. This was washed with 500 to 600 ml of 2 × SSC in order to remove all traces of contaminating UV-absorbing material. Nitrocellulose chromatography was performed at room temperature.

Preparation of plasmid DNA: Circular DNA was prepared by a modification of the method used for φX174 RF.[11-13] A plasmid-positive strain of *S. aureus* was grown to stationary phase in 500 ml of CY medium.[1] The cells were centrifuged and washed with four separate 50-ml portions of cold 0.15 *M* EDTA, pH 7.0. The washed cells were then suspended and frozen in 50 ml of the same buffer, and lysed by thawing in the presence of 50 μg/ml lysostaphin. The extremely viscous lysate (room temperature) was brought to pH 12.3 with 1 *N* NaOH and incubated at this pH with vigorous stirring for 3 min. Due to the high viscosity of the lysate, pH equilibration was poor; as long as 5 min was often required to attain a steady value of 12.3. Upon neutralization with 6 *N* HCl (final pH, 7), a heavy precipitate was formed; this precipitate was removed by centrifugation in the cold. The supernatant (*ca.* 65 ml) was then incubated with RNase (10 μg/ml final concentration) at 80° for 3 min, and after removal of the resulting precipitate by centrifugation (5°) was chromatographed on a 500-ml Sephadex G-100 column (5°) with 2 × SSC as the elution buffer. Fractions containing excluded material were combined and passed through a nitrocellulose column. The eluate, containing circular duplex DNA, was concentrated by rotary flash evaporation at 30°. The average yield per 500 ml of culture was 100 μg circular DNA of which 80 to 100% was form I.

Preparation of phage DNA: Phage P11 was grown in lysing broth[9] on strain RN450, sedimented by centrifugation, resuspended in phage buffer,[9] and purified by isopycnic banding in CsCl of density 1.450 gm/ml. The purified phage was dialyzed, and the DNA extracted with phenol. After dialysis against 0.1 × SSC, the DNA was examined by electron microscopy.

Electron microscopy: The plasmids and φX174 RF preparations were converted to the relaxed forms by X-irradiation. Samples were spread as previously described,[11] with the following modifications: RFII and plasmid II were present in about equal amounts (number basis) in the spreading solution, which contained, in addition, 0.025 *M* EDTA. Specimens were usually stained,[14] rotary shadowed with Pt-Pd, and examined with an Elmiskop 1A electron microscope at instrumental magnifications of 3600 or 10,000. Metal-shadowed carbon replicas of diffraction gratings (E. F. Fullam, Inc., and Ladd Industries, Inc.) were used as magnification standards. The position of the objective lens current control dial was noted for each specimen area photographed. After a grid had been examined and removed, the magnification standard was photographed, and the change in objective lens current required to refocus the replica was related to the change in focal length. This procedure permits correction of the magnification for variation of specimen position. The maximum pincushion distortion, measured in the radial direction at the corner of the photographic plate, was about 1–2% at the two magnifications used. Molecules selected for measurements were obtained from the central rectangular region comprising about 60% of the area of the plate; within this region there was no detectable distortion. Only completely open molecules were selected for measurement. Images were enlarged photographically or by tracing their projections on a paper screen. In either case, the negative was positioned in the enlarger or projector so that the image was projected onto a central distortion-free area. The contour lengths of the enlargements were measured with a map measure (Keufel and Esser, 630320). These precautions allowed the calculation of contour lengths with greater precision than previously attained.[11]

Ultracentrifugation: Ethidium bromide-CsCl preparative centrifugations and analytical zone sedimentations were performed as previously described.[11, 12] Buoyant densities

were determined at 48,000 rpm with denatured DNA of *Micrococcus luteus* (ρ = 1.742 gm/ml) or ϕX174-RF (ρ = 1.707 gm/ml) as a marker.[15]

Results.—Preparation and properties of plasmid DNA: The preparative procedure is based on the resistance of covalently closed circular duplex DNA to alkali denaturation. With slight modification we have found this technique to be generally applicable to the preparation of circular DNA from a variety of sources, the largest molecule thus far isolated having a molecular weight of 25×10^6 daltons and the smallest 1×10^6 daltons.[16] The penicillinase-plasmid preparations described here were 80–100 per cent form I, showed a single sharp peak with a buoyant density of 1.690 gm/ml in CsCl density gradient centrifugation, and could be resolved into pure form I and II by ethidium bromide CsCl density gradient centrifugation.[17, 18] An electron micrograph confirming the circularity of these molecules is shown in Figure 2*A*.

Molecular weight of the wild-type plasmid and deletion mutants: Contour lengths and calculated molecular weights of circular DNA molecules isolated from four staphylococcal strains, including two with plasmid deletions, are given in Table 1. A correlation between contour length and extent of deletion is illustrated in the form of a genetic map in Figure 1. Also included in Figure 1 is the contour length of P11*de*, which is greater than that of the wild-type plasmid, but not as great as that of P11 phage DNA (14.4 μ).

Complex forms of plasmid DNA: Although any single plasmid preparation appears homogeneous by zone sedimentation, electron microscopic observations indicate the presence of circular dimer and catenated forms. Thus, the wild-type plasmid (PI_{258}) as isolated from strain RN453 contains about 0.8 per cent circular dimers and 1.7 per cent catenated dimers. A total of 1185 molecules was scored and classified according to the procedure of Clayton *et al.*[20] Selected electron micrographs of these multiple-length forms are shown in Figure 2*B*, *C*, and *D*.

Discussion.—Our conclusion identifying DNA rings isolated from certain strains of *S. aureus* with penicillinase plasmid genomes is based on two considerations: (1) We have been unable to obtain circular DNA from a plasmid-negative derivative of the strains examined, and (2) the contour lengths of the DNA species isolated are proportional to the genetic lengths of the corresponding plasmids. Based on a molecular weight for ϕX174 RF of 3.4×10^6 daltons, our measurements of the wild type and two deleted plasmids correspond to molecular weights of 18.6×10^6, 16.4×10^6, and 15.3×10^6 daltons, respectively. These two deletions, which are partially overlapping, together account for nine of the eleven known plasmid markers (see Fig. 1). Since the larger deleted plasmid lacks six known cistrons and the smaller lacks eight, the average size of each of these cistrons is about 600 nucleotide pairs. On the basis of this average it appears that the nine cistrons involve no more than one fourth of the plasmid molecule. The only plasmid cistron whose product is known is that for penicillinase, an enzyme consisting of a single polypeptide chain of 256 amino acid residues.[22] This length is not inconsistent with an average cistron of 600 nucleotide pairs. If the other deleted cistrons tend to be of about the same size, the missing DNA would include little else. If, as appears to be the case,[6] the deletions are

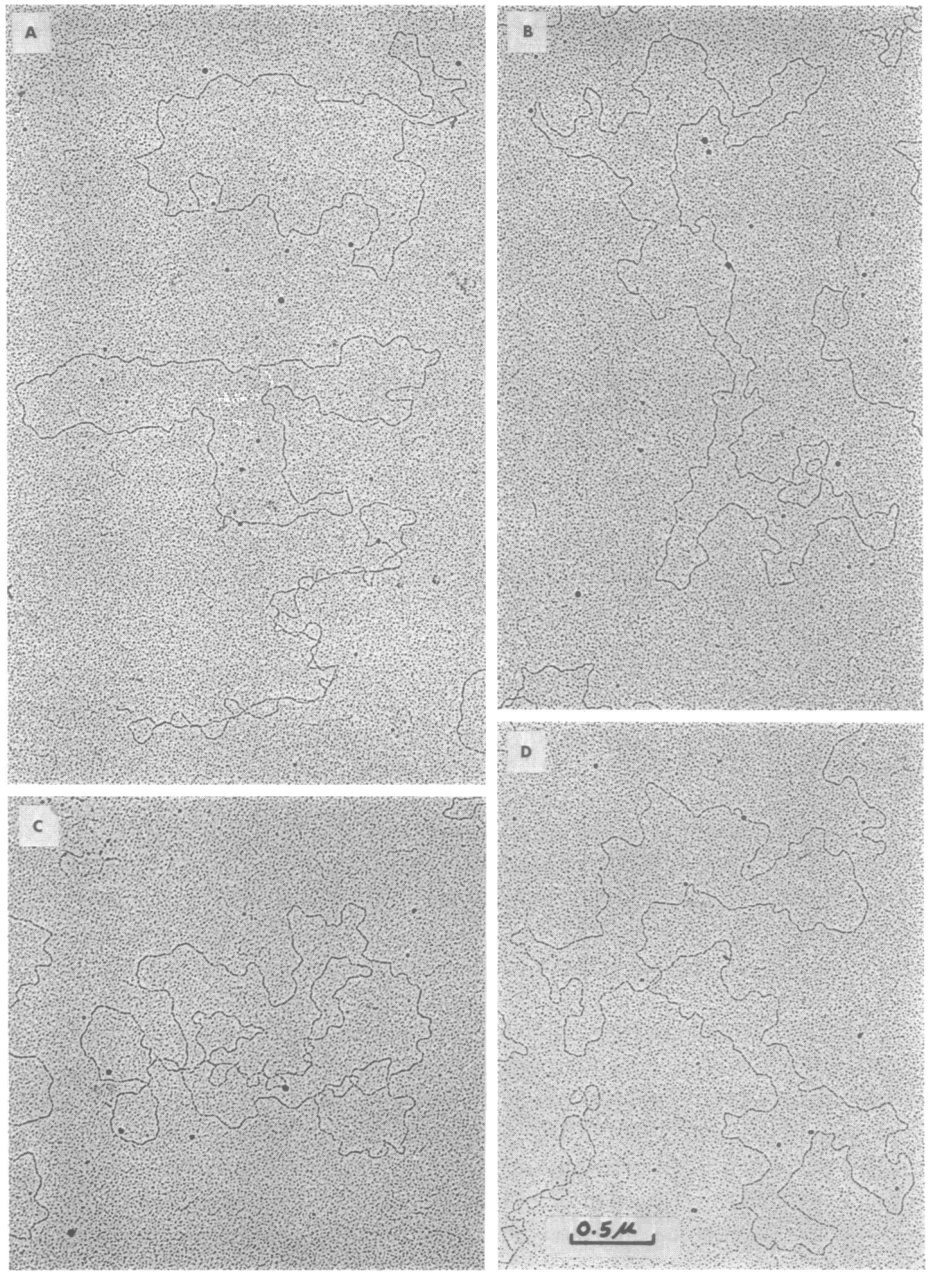

FIG. 2.—Electron micrographs showing selected fields of the wild-type plasmid (PI_{258}). The DNA was stained with uranyl acetate in acetone and then rotary shadowed with Pt–Pd. (*A*) Normal-length rings, (*B*) field containing a circular dimer, (*C* and *D*) fields containing catenated dimers.

TABLE 1. *Contour lengths and estimated molecular weights of the wild-type plasmid, two deletion mutants, and P11de.*

	Plasmid Molecules		φX174 RF Molecules		
Plasmid type	Number measured	Mean contour length and standard deviation (μ)	Number measured	Mean contour length and standard deviation (μ)	Relative molecular weight of plasmid (daltons)
PI_{258}	15	9.41 ± 0.05	15	1.72 ± 0.02	18.6×10^6
PI_{147} *ero* 15	15	8.05 ± 0.14	15	1.67 ± 0.03	16.4×10^6
PI_{258} *pen* 102	15	7.56 ± 0.05	15	1.68 ± 0.02	15.3×10^6
P11*de*	2	12.2 ± 0.07	0	...	...

Form II of φX174 RF was included in each spreading solution as an internal contour length standard. The molecular weight of each plasmid was calculated from the ratio of its contour length to that of φX174 RF in the same field. The molecular weight of the latter is assumed to be 3.4×10^6 daltons.[19]

continuous, the cistrons involved would have to be adjacent to one another, as indicated on Figure 1.

Transducing particles for two of the four elements studied, PI_{258} and P11*de*, have been found to have the same buoyant density and sedimentation rate as P11 plaque-forming particles,[21] indicating that the phage heads contain a fixed amount of DNA. Since all of the elements studied are smaller than the P11 genome (molecular weight about 28×10^6 daltons), the transducing particles might also contain phage DNA fragments, host chromosome fragments, or redundant or multiple plasmids.

In conclusion, the isolation of plasmid DNA promises to aid greatly our genetic and biochemical studies aimed at understanding both the mechanism of generalized transduction and the control of plasmid replication.

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[10] The abbreviations used are: SSC, 0.15 *M* NaCl, 0.015 *M* sodium citrate; RF, replicative form; form I, covalently-closed circular DNA; form II, circular DNA containing one or more single strand breaks. For gene terminology see the legend for Figure 1.

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